Original Articles

Progesterone receptor expression by human leukocyte cell lines: molecular mechanisms of cytokine suppression

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Summary

Purpose: We investigated progesterone (P) signaling in human leukemia cells, shown to downregulate cytokines with P.

Methods: The following tests were utilized: PCR with FAM labeled primers and Gene Scan with the Applied Biosystems 373 DNA sequencer for progesterone receptor (PR) mRNA, immunohistochemistry using monoclonal antibodies (Zymed and Ventana) for PR protein, RT-PCR for glucocorticoid receptor (GR), NFκB (p65, p50, p52), c-rel, IκB-α, c-jun, c-fos, mRNA, transient transfections with pNF-κB-SEAP, and pGRE-SEAP vectors with chemiluminescence detection for NFκB and GR activity.

Results: PR transcripts were demonstrated in MOT, U937, K562, THP-1, 8226, U226, not in JUKAT, HL60, HUT102, isolated normal peripheral blood mononuclear cells, or purified CD4+ or CD8+ T cells. Estradiol increased PR mRNA in MOT and U937. MOT, U937, K562, KG-1, 8226, ATL, and CD8+HTLV-1 expressed PR protein. SRIH-BATL, 729PH6NEO, HS-1, R-CLL, and JURKAT were negative. Steady state mRNA for GR, NFκB (p65, p50, p52), IκB-α, c-jun were unchanged with P in MOT and U937; c-fos and c-rel were not detected. There was a concentration-dependent reduction of NFκB activity with P in MOT and U937.

Conclusion: The mechanism of cytokine suppression by P is mediated at least in part by suppression of NFκB, but the interaction of sex hormones, receptors, and transcription factors is complex.

Kew words: Reproductive immunology; Cytokines; Second messengers; Transcription factors.

Introduction

Progesterone is an immunosuppressive hormone [1-14]. High serum levels (10^{-6} M) during pregnancy are associated with an increased risk of infection with T cell dependent organisms, such as *Candida albicans* [15]. Progesterone is critical for tolerance of the fetus and successful pregnancy outcome [15, 16]. Fluctuations of levels, as occurs in a normal cycle, may alter susceptibility to sexually transmitted disease, including HIV [1, 5, 10]. The temporary remission of several autoimmune diseases with pregnancy, including rheumatoid arthritis and some patients with inflammatory bowel disease, and the variation in symptoms with the menstrual cycle, further support progesterone's activity as an anti-inflammatory agent [7, 9, 10]. Previous researchers have documented multiple direct effects on the function of immune cells [2, 9, 17]. Progesterone (P) decreases cytokine production, including IL-8, TNF-β, IL-6, GM-CSF, MIP-1α, GRO-α, RANTES, TNF-α, and IL-10 in various cells [2, 4, 16]. Progesterone also decreases inducible nitric oxide synthase and nitrous oxide, monocyte and macrophage proliferation, and giant cell formation, and increases prostaglandin E2 production. In T cells, progesterone inhibits proliferation, blocks activation, decreases the cytotoxic response and the mixed lymphocyte reaction, alters CD30 expression, lowers thymic weight, and decreases chemokine receptor expression [2, 8-10, 16]. We previously determined [2] the effect of progesterone on the cytokine profiles of major immune cell lineages using T cell lines (MOT, SRIK-NKL), B cell lines (SRIS-BAML, 729PH6neo), and myeloid cell lines (U937, HL-60). Cytokine production, including several chemokines, was decreased by 40-90% in MOT and U937 cells, the most sensitive to down-regulation. The molecular mechanisms of progesterone-induced immunosuppression are not known, and controversy exists in the literature regarding the expression of progesterone receptor (PR) by human immune cells [5-7, 9, 18-26]. There is evidence that PR may be induced in gamma/delta T cells by allogenic stimuli [21].

The expression of human PR in cells known to be sensitive to P, such as breast and endometrial epithelia, is controlled by two promoters that direct the synthesis of mRNA transcripts encoding 120 kD PR-β and 90 kD PR-α [6, 7, 9, 27-32]. These isoforms are identical except that the smaller PR-α is truncated, missing the N-terminal 164 amino acids. The PR-α and PR-β proteins differ in their capacity to activate target genes. Their relative amounts also vary in tumors, including breast carcinomas, and can be a determinant of P responsiveness, affecting treatment and prognosis [33-38]. PR-α has been reported to act as a transdominant inhibitor of PR-β, and of the transcriptional activities of receptors for glucocorticoids, mineralocorticoids, androgens, and estrogens [37-39]. A third PR, termed PR-C (78 kD) [28, 30], and a fourth (60 kD) [40, 41], have also been found. There is even greater potential complexity, as nine or more functional PR mRNA transcripts have been detected in normal tissues, breast cancer cells, as well as in cell lines containing deletions of various exons [34, 40, 41].

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PR is a member of a superfamily of ligand-activated transcription factors. Upon hormone binding, PR dimerizes, undergoes phosphorylation, and binds to specific DNA sequences, progesterone response elements (PRE) in the 5'flanking regions of progesterone-responsive genes [1-11]. Depending on the target cell, target gene, and receptor type, up-regulation or down-regulation of transcription may result. However, progesterone has a multitude of effects, including inhibition of cytokines such as TNF-α and chemokines, on proteins encoded by genes that do not possess PRE. For example, many cytokine genes are regulated by transcription factors including NFκB and AP-1 [42, 43] and glucocorticoid receptors [7, 44]. The potential for interaction of PR with these and other transcription factors, co-regulatory proteins, co-activators/co-repressors, and nuclear receptors in human immune cells is not known, but could be a mechanism to explain the immunosuppressive effects of progesterone [6, 7, 45].

The transcription factors NFκB [42] and AP-1 [43] control the expression of many genes critical to the immune response, including cytokines, cytokine receptors, immunoglobulins, enzymes (cyclo-oxygenase, nitric oxide synthase), and adhesion molecules. These transcription factors have been shown to be targets of several anti-inflammatory agents, including glucocorticoids, aminosalicylates, and bile acids. AP-1 is a DNA binding protein present in many cell types, including leukocytes. AP-1 is functional as dimers of different component proteins from the c-fos and c-jun families of proto-oncogenes, bound together in leucine zipper formation. AP-1 binds to the DNA consensus sequence TGACTCA and increases inflammatory cytokines (i.e., interferon-γ and IL-2) and metalloproteinase production. This binding is critical for cell differentiation, activation, cytokine profile determination, and immunoglobulin production. Decreased AP-1 activity is central to the process of T cell anergy and tolerance.

NFκB, like AP-1, is also a dimer, composed of different combinations of the Rel/NFκB family of proteins, which include NFκB p105/p50, $NF\kappa B_2$ (p100, p52), Rel A (p65), Rel B, and C Rel. The potent NFκB Rel A/p50 dimer is present in the cytoplasm of most human cells, including immune cells, but is usually inactive because it is bound to its inhibitor IκB (α or β). During an inflammatory response, such as occurs in infection or inflammatory disease, cytokines, bacterial/viral products, and other stimuli can cause IκB to be phosphorylated, ubiquinated, and degraded. This releases NFκB (p65), allowing it to translocate to the nucleus and upregulated cytokine receptors, and adhesion molecules, such as ICAM-1. As with AP-1, decreased levels of NFκB are associated with CD4+ T cell anergy [9, 10, 18, 45].

Glucocorticoids down-regulate AP-1 DNA binding via the formation of protein complexes of nuclear glucocorticoid receptor (GR) and AP-1 [10]. NFκB is inhibited by glucocorticoids by distinct mechanisms depending on cell type. In some cells, the activated GR physically binds to p65, preventing DNA binding and transcriptional activation. In other cell types GR acts to up-regulate IκB-α synthesis, increasing the concentration of its natural inhibitor and terminating NFκB action. Sex steroid receptors have also been shown to utilize these transcription factors as downstream signaling pathways in specific non-immune cell types well-known to express PRs, estrogen receptors (ERs), or androgen receptors (ARs). The effect of progesterone on these final common pro-inflammatory pathways in human immune cells is not known.

In this study, we used cell lines MOT and U937, producing significant amounts of cytokines important in mucosal immunity, and found by us to be most sensitive to cytokine down-regulation by progesterone [2, 46, 47] to determine the presence or absence of PR, and explore molecular mechanisms of immunosuppressive action.

Materials and Methods

Drug preparation: Progesterone and estradiol (Sigma) were dissolved in ethanol and diluted with RPMI-1640 5% FCS, 1% glutamine to a final concentration of 0.5-50 μM (5 μM for estradiol), within physiologic range, and nontoxic by Trypan blue exclusion test. The final concentration of ethanol (0.5% maximal) was shown not to alter cytokine production in another of our studies [2]. Dexamethasone for injection, 4 mg/ml, was diluted to a final 5 μg/ml with RPMI-1640 5% FCS 1% glutamine.

Cell lines: HL60, U937, SRIS-BAML, 729PH6neo, MOT and SRIK-NKL cells were used for the initial screen of progesterone effects on cytokine profiles in our previous study [2]. MOT, U937, MCF-7, JURKAT, K562, THP-1, HL60, HUT-102, RPMI 8226, U266, normal peripheral blood mononuclear cells isolated with Hypaque-Ficoll, and purified CD4+ and CD8+ T cell (Dynabead) were tested for PR mRNA by GeneScan. MCF-7, U937, KG-1, K562, MOT, SRIC-ATL, FC36.22 (HTLV-1+ CD8+), JURKAT, R-CLL, RPMI 8226, U226, HS-1, 729PH6neo, and SRIH-BATL were stained for PR protein as described below. MCF-7 is a hormone-responsive breast carcinoma cell line known to be PR and ER positive. THP-1 is monoblastic, KG-1 myeloblastic, K562 CML-blastic, and HL60 myeloblastic. HUT-102 is a HTLV1+ CD4+ T cell line, SRIC-ATL is a HTLV1+ CD4+ T cell line, FC36.22 is a HTLV-1+CD8+ T cell line, and JURKAT is a CD4+ T cell line. R-CLL is a B-CLL line, RPMI-8226 myeloma, U226 myeloma, HS-1 is a HTLV-1+ B cell line, 729PH6neo is a HTLV-2+ B cell, and SRIH-BATL a HTLV1+ B cell line [2, 46, 47]. All cells were maintained in RPMI-1640 5% FCS 1% glutamine without antibiotics. MCF-7 was grown until confluent, and harvested after trypsinization.

Immunohistochemical detection of PR protein: Progesterone receptor protein in U937, MOT, and other cell lines was examined by standard immunohistochemical techniques using Ventana (Tucson, AZ), an automated immunohistochemical slide staining device Model 320. PR mouse monoclonal antibodies from Ventana (clone PGP-1AG) were directed against ligand-binding sites present in the human PR. PR mouse monoclonal antibody (clone PR-2C5) from Zymed Lab Inc. (San Francisco, CA), which was directed against the peptide representing N-terminal region of PR starting around amino acid 500, was also used. Both antibodies detected PR-α, PR-β, as well as truncated PR-α proteins. Isotype-matched control antibodies served as negative antibody controls. MCF-7 cells and sections of hormone sensitive breast carcinoma were used as

known positives for PR proteins. PR expression in U937 and MOT were also examined with and without 16-24 hours incubation with 25 μM and 50 μM progesterone or 2.5 and 5 μM β-estradiol to investigate modulation of PR protein expression by sex steroids.

RNA extraction and RT-PCR for steroid receptors: Total RNA was isolated from untreated U937, MOT, MCF-7 (known PR positive), and other cells using the Qiagen RNAeasy extraction kit, treated for 30 minutes at 37°C with RNase free DNase (1 unit/μg RNA) and RNase inhibitor (10-20 units), heated for 30 minutes at 70°C to inactivate DNase and used in RT-PCR assays [48], RT-PCR sense primer 5'-GTCTTAATCAACTAGGC-GAG-3'. Positions 2149-2168 and anti-sense primer 5'-GTTTCACCATCCCTGCCAATATCT-3' positions 2755-2778 were, according to Iwai *et al.* [49], from the c-terminal region of PR with an expected PCR product of about 630 bp. RT of RNA and PCR amplification of cDNA were performed [48] using a Gene Amp EZ rTth RNA PCR kit (Perkin Elmer, Foster City, CA): a single tube, single buffer system and recombinant *Thermus thermophilus* DNA polymerase, which also acts as a thermostable DNA polymerase. The reaction mixture for RT of RNA and PCR amplification of cDNA contained in 0.2 ml reaction tubes, 50 mM Bicine, 115 mM K acetate, 8% w/v glycerol pH 8.2, 2.5 mM $Mn(OAC)_2$, 300 μM each of dATP, dCTP, dGTP, and dTTP, 5 units of rTth DNA polymerase, 0.45 μ M of each of the primer pairs, approximately 1-2 μg of total RNA in a final volume of 50 μl. The MJ Research PTC-100 thermal cycler with a heated lid was used with RT for 30 minutes at 60°C followed by 90 seconds at 94°C and then 40 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final 8-minute extension at 72°C linked to a soak cycle at 4°C. The primer pairs for β-actin from R&D Systems (St. Paul, MN), which gave a PCR product of 528 bp, were used as external or internal controls. The PCR products were examined by agarose gel electrophoresis and ethidium bromide staining and documented by UV photography according to the procedure previously described [48]. Out of several T,B, myeloid, and monocytoid cell lines examined, including MOT and U937, none gave any band detectable by ethidium bromide except for actin control, whereas the MCF-7 cell line gave the expected 645 bp band.

The 645 bp product for the MCF-7 cell line was recovered from the gel using Qiagen quick gel extraction kit and analyzed using the ABI Prism 377 DNA Sequencer and their protocol to confirm that the nucleotide sequence of the product was identical to that of the published sequence of PR. Since the PCR product for PR was not detected for any other cell line except MCF-7, another set of primer pairs were used, PR sense 5'-CCATGTGGGAGATCCCCACAGGAGTT-3' and PR antisense 5'-TGGAAATTCAACACTCAGTGCCCGG-3' with a 320 bp product in the E domain of PR [50]. Again, RT-PCR products for PR were detected by ethidium bromide staining for MCF-7 and a genital tract epithelial cell line positive for PR (kindly provided by Dr. R. Fichorova), but not for U937, MOT, or other hematopoietic cell lines.

Thus, to increase the sensitivity of the detection of products, FAM labeled PR sense primers and unlabeled antisense primer [49] were used in RT-PCR as given above. The PCR products were analyzed using the GeneScan Applied Biosystems Inc. (Foster City, CA) 373 DNA Sequencer according to their protocol. Also, using this technique, PR mRNA modulation by sex steroids in U937, MOT, and JURKAT was examined in cells that had been incubated with and without 5 μM β-estradiol or progesterone 5 μM for 16-24 hours. Cultured cells were centrifuged for 10 min at 3000 g, supernatant was discarded, and cells were resuspended in fresh medium. The cells at $0.5x10^6$ cells/ml were then incubated with and without drugs in 24 well plates for the given time period. Subsequently, well contents were aspirated, and centrifuged for 2 min in a microcentrifuge. The supernatant was discarded and RNA extracted as for untreated cells; the above protocol was followed.

In addition to examining for PR, the expression of mRNA for the glucorticoid receptor (GR) was also examined in U937, MOT, and other cell lines using the following primers: GR sense 5'-TGCAGCAGTGAAATGGGAAA-3' and GR anti-sense 5'-GGGAATTCAATACTCATGGTA-3' giving a 533 bp product covering bp 1838-2371 in the hormone domain of GR [51]. There is little homology between GR, PR and MR at this point. RT-PCR conditions, agarose gel electrophoresis, and ethidium bromide staining were performed as described above for PR.

RT-PCR for NFκB and AP-1 expression and modulation by progesterone: Total RNA from U937 and MOT cells that were cultured in 24 well plates, at 0.5 x 10^6 cells/ml in RPMI-1640 5% FCS glutamine with and without 50 μ M progesterone for 15 minutes to 72 hours, was extracted using the Qiagen kit, treated briefly with RNase free DNase and RNase inhibitor, heated to inactivate DNase and used in RT-PCR assays as described above. Primer pairs for c-fos (612 bp product) and c-jun (409 bp product) were from Clontech (Palo Alto, CA). Primer pairs for NFκB (p65, p50, p52), c-Rel, IκB-α were according to Inoue *et al.* [52] and gave PCR products of 223 bp, 262 bp, 220 bp, 285 bp, and 218 bp, respectively. Primers for β-actin (528 bp product) from R&D Systems were used as internal or external controls. RT-PCR conditions were the same as described above for PR except that amplification cycles were varied between 25-40 to evaluate changes in product accumulation relative to actin. The PCR products were examined by agarose gel electrophoresis and ethidium bromide staining and documented by UV photography on Polaroid #57 film, according to the procedure previously described [49].

Modulation of GRE and NFκB transcription factor activity by progesterone: Secreted alkaline phosphatase (SEAP) receptor vectors for nuclear factor κB (NFκB) and glucocorticoid response element (GRE) from Clontech (Palo Alto, CA) were used to transiently transfect U937 and MOT cells, followed by incubation of cells without and with drugs, and assay of SEAP activity to quantitatively determine the effect of the drug on the activity of these important transcription factors. To determine the effect of progesterone (5 μ M and 50 μ M), dexamethasone (5 μg/ml), or progesterone plus dexamethasone, $1x10^5$ cells in 150 μl of RPMI 1640 and 5% fetal calf serum were transfected with 0.15 μg vector DNA using 50 μl condensing buffer, 1.2 μl enhancer, 4 μl Effectine (Qiagen, Inc.) and 200 μl RPMI medium in 24 well plates. After incubation at 37°C for 36 hours for U937 and for 48 hours for MOT, 10 μl of medium, progesterone, dexamethasone, or progesterone plus dexamethasone were added and the incubation continued. The culture supernatants were collected at various intervals after drug addition by centrifugation (8000x G) of the well contents and assayed at least in triplicate for SEAP. Clontech's Great ESCAPe SEAP chemiluminescence detection kit with luminometer was used to assay secreted alkaline phosphatase in the supernatants according to the protocol.

In addition, modulation of activated NK-κB p65 by P in MOT and U937 cells was also determined using the ELISA-based Trans AM NF-κB p65 kit from Active Motif (Carlsbad, CA). Here MOT or U937 cells (105 cells/ml in RPMI 1640 5% fetal calf serum) were incubated without or with 5 μM or 50 μM P in triplicate. At one hour, six hours, 22 hours, and 24 hours the cells were removed and whole cell extracts were prepared as recommended by Active Motif. Extracts containing 10 μg protein were used for the quantitation of activated NF-κB p65

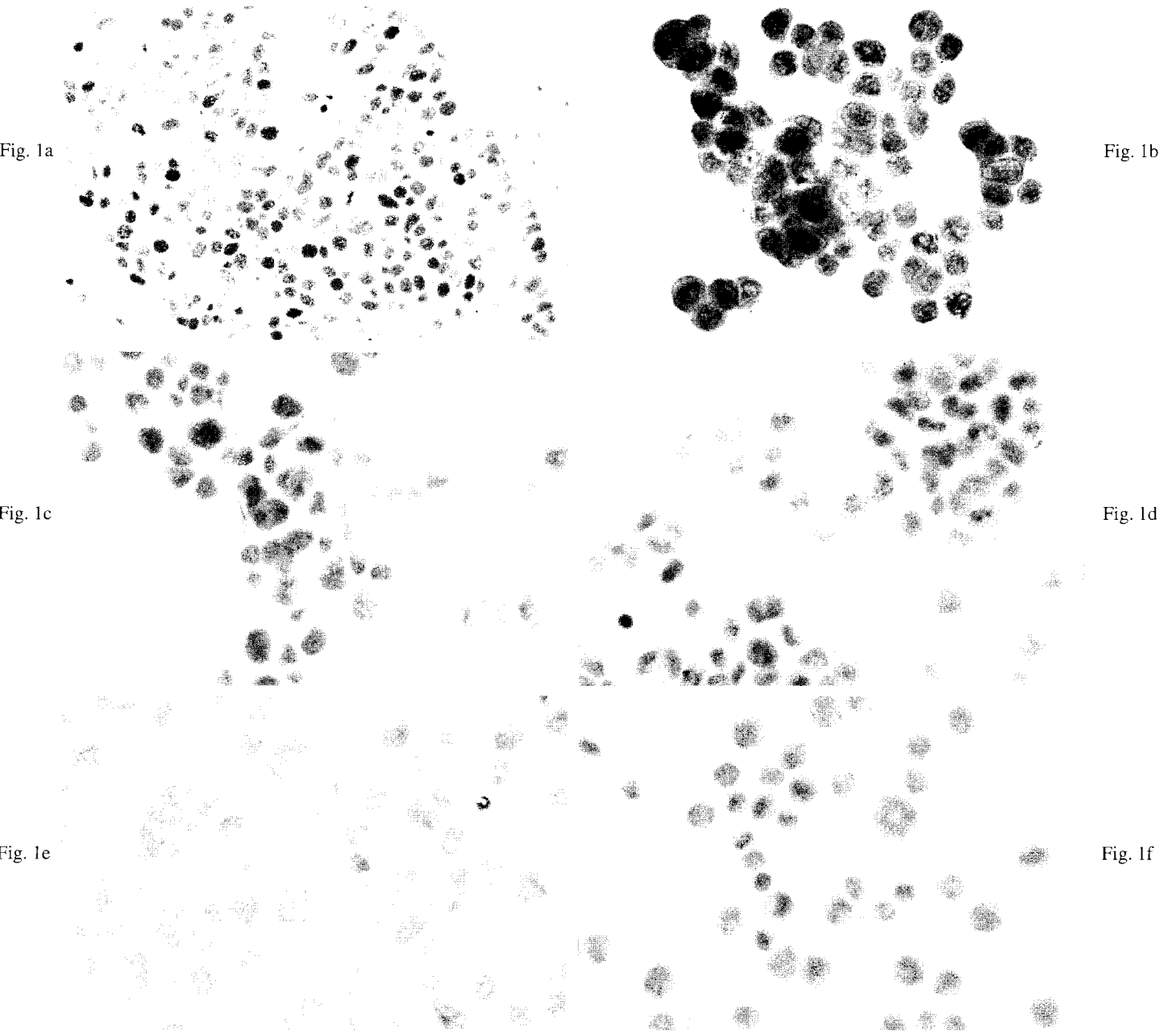

Figure 1 (a-f). — Immunohistochemical staining using Ventana antibodies for PR protein in MOT, U937, MCF-7, breast carcinoma cells (positive control), and negative control (MOT and U937 stained with isotype matched control antibodies). 1a depicts positive controls breast cancer biopsy and 1b MCF-7 cells. 1c shows results in MOT, and 1d U937, with nuclear staining demonstrating PR presence in many but not all cells. 1e and 1f depict MOT and U937 negative controls, respectively.

using the Active Motif kit and protocol. Whole cell extract from activated HeLa cells in the kit was used as the standard and the activity was expressed as absorbance at 450 nm.

Results

Progesterone receptor expression: Using immunohistochemistry techniques with Ventana and Zymed antibodies that bind to the hormone-binding region and N terminal of the PR respectively, receptor protein in MOT and U937 was examined. Results with Ventana antibodies are given in Figures 1a-1f. Positive controls of the breast cancer biopsy specimen and MCF-7 breast carcinoma cell lines are pictured in Figures 1a and 1b, negative controls (MOT and U937) stained with isotype matched control antibodies, are shown in Figures 1e and 1f. Most MOT cells (Figure 1c) showed strong staining present in the nucleus, although there was some variability, and a few cells had no detectable staining. U937 cells (Figure 1d) also showed some nuclear staining, again with some variability. Thus, both MOT and U937 cells can and do express progesterone receptor proteins. However, the

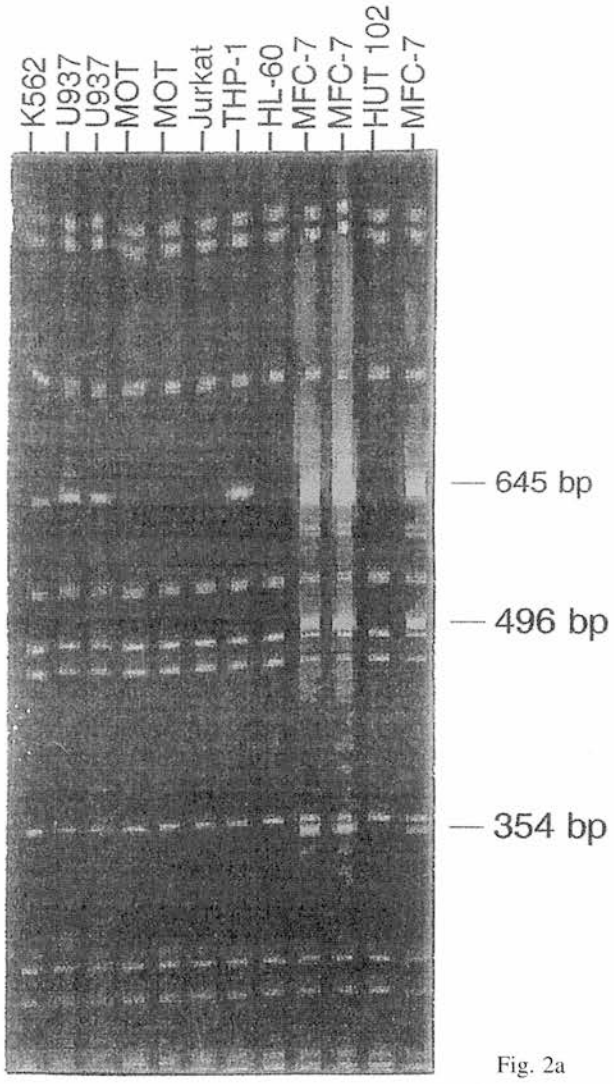

Fig. 2a

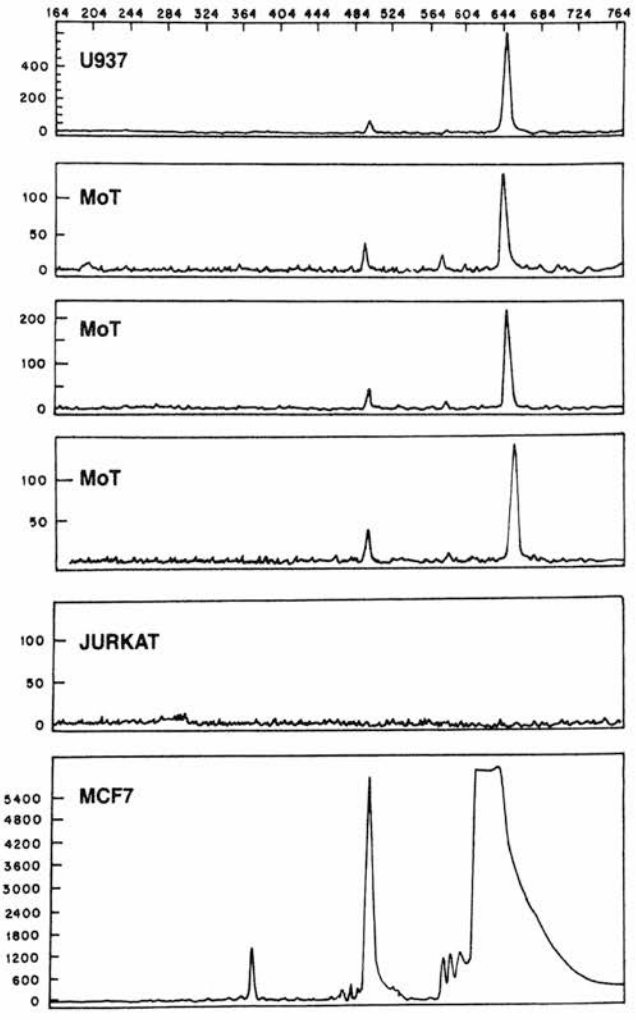

Fig. 2b

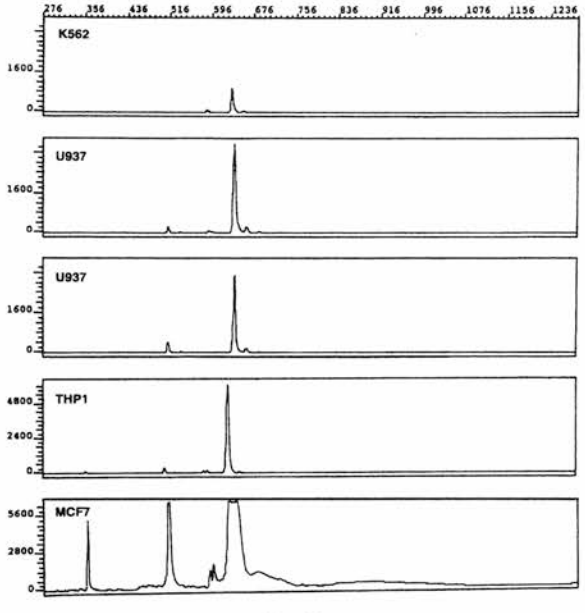

Fig. 2c

Figure 2. — Representative scan (2a) and computer analysis (2b and 2c) of gene scan results demonstrating presence of PR mRNA in K562, U937, THP-1, MOT, and MCF-7 cell lines. Several products were detected in MOT, U937, and MCF-7, and THP-1, including the expected fragment size of 645 bp. PR mRNA was not detected in JURKAT. MCF-7 expressed large amounts of products of 496 and 354 bases as well.

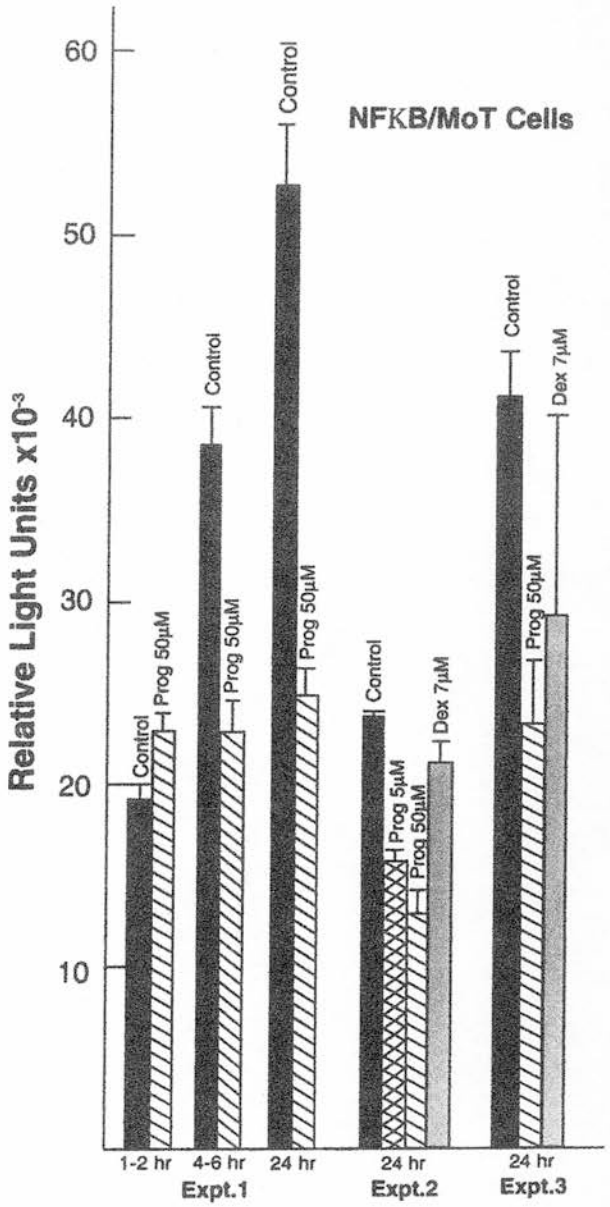

Fig. 3a

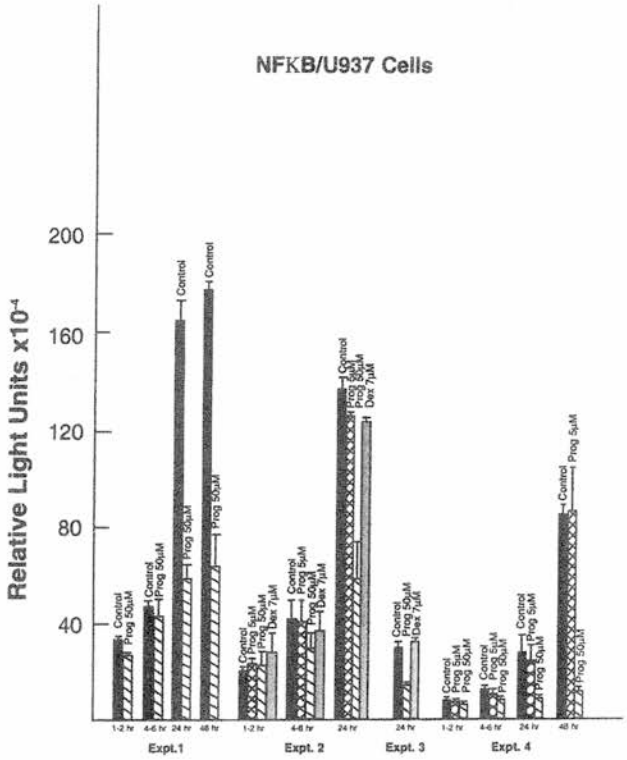

Fig. 3b

Figure 3. — Modulation by P of NF-κB activity in MOT (Figure 3a) and U937 (Figure 3b) cells. Results of transient transfections with reporter plasmid for NF-κB expressed in relative light units.

individual differences in PR expression by MOT and U937 cells suggested the existence of variably sensitive and insensitive subpopulations. Given these findings, additional cell lines were stained (Table 1). KG-1 and K562 myeloid cells stained positive, as did HTLV-1 positive T cells SRIC-ATL and FC36.22. Multiple myeloma cell lines U226 and RPMI-8226 were also positive. Staining for PR could not be detected in JURKAT, R-CLL, HS-1, 729PH6neo, or SRISB-ATL. In other experiments, treatment with 5-50 μM progesterone for up to 24 hours did not detectably affect MOT or U937 PR protein, but it increased in both after incubation with 5 μM estradiol (not shown).

Progesterone receptor mRNA expression: To further examine the cell lines for the ability to express PR, mRNA was extracted and subjected to standard RT-PCR using different PR primer sets, with MCF-7 breast carcinoma cell lines, and genital tract epithelial cell lines (kindly provided by Dr. R. Fichorova) as positive controls. PR mRNA was detectable in the positive controls, but was not detected in MOT and U937 (not shown). Treatment with 5-50 μ M P in MOT and U937, or differentiation of U937 with TPA, did not lead to the detection of PR mRNA. Using the FAM labeled primers and Gene Scan with Applied Biosystems 373 DNA Sequencer, however, PR mRNA was detected in MOT, U937, and massive amounts in MCF-7 (Figures 2a-2c). The scan of the gel demonstrated the presence of several transcripts of 645, 546, 496, and 354 base fragments sizes in MCF-7. The highly expressed 645 base fragment was also prominent on gel scans for K562, U937, and monoblastic THP-1 cells (Figure 2a). Computer analysis (Figure 2b and Figure 2c) revealed the presence of PR transcripts of the

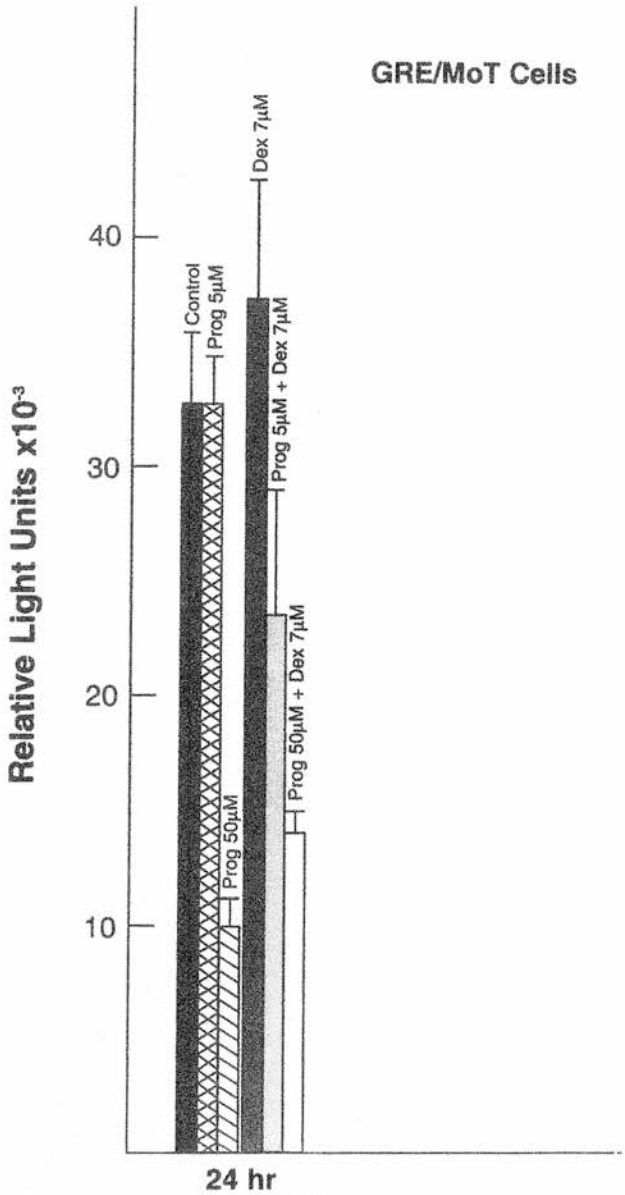

Fig. 4a

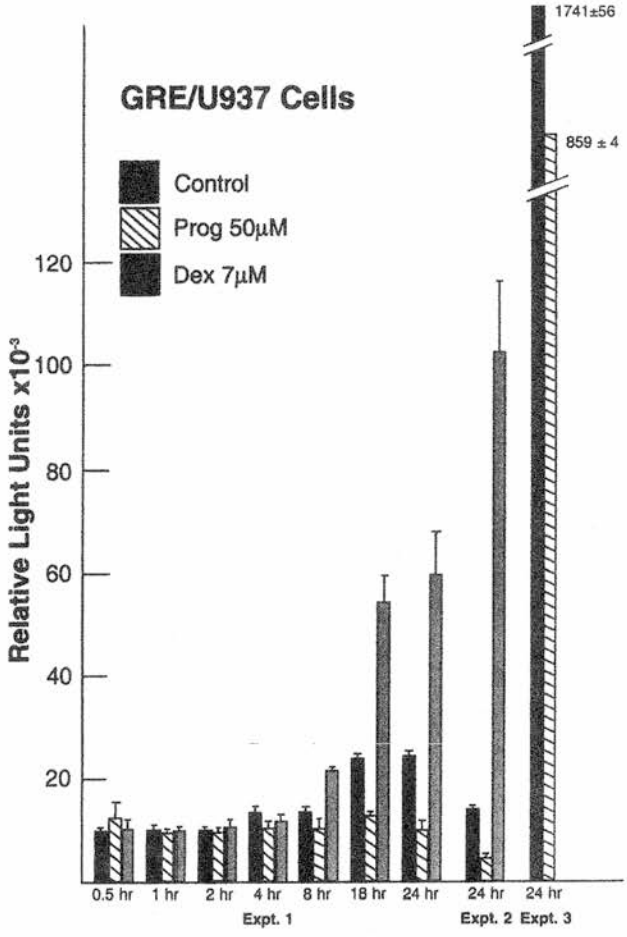

Fig. 4b

Figure 4. — Modulation by dexamethasone and P of GR activity in MOT (Figure 4a) and U937 (Figure 4b) cells. Results of transient transfections with reporter plasmid for GR, expressed in relative light units.

above sizes in MOT, as well as U937, MCF-7, K562, THP-1. In subsequent experiments, PR mRNA expression with multiple transcripts was also detected in RPMI-8226 and U226 myeloma cell lines by FAM-labeled primers (Table 1) but not in JURKAT cells. Several MOT and U937 samples analyzed demonstrated reproducibility of PR mRNA (Figure 2b and Figure 2c). Furthermore, treatment of MOT and U937, but not JURKAT cells with 2.5-5 μM β-estradiol further increased the amount of transcript detected (not shown). These results support the existence of multiple transcripts, which if translated, could result in unique progesterone receptor isoforms with variable agonist/antagonist activities, and further modify progesterone effects on immune cells. Expression may also be inducible by other steroid hormones, or by immune cell activation, as with viral infection or malignant transformation. As myeloid/monoblast, T, and B lineage (multiple myelomas) lines were found to be positive (Table 1), PR expression by all the major immune cell types is again supported.

Transcription factor mRNA expression and modulation by progesterone: RT-PCR studies showed that MOT and U937 had identical constitutive transcription factor profiles, expressing GR, NFκB (p65, p50, p52), c-jun, and IκB-α mRNA. The c-Rel component NFκB transcription factor was not detected, nor was the c-fos component of AP-1. Treatment with P up to 50 μM did not detectably alter this steady-state RNA expression of GR, NFκB (p65, p50, p52), c-jun, or IκB-α in either MOT or U937 (data not shown). This suggested that cytokine modulation by progesterone did not involve alteration in the amount of component mRNA's of these key transcription factors. As c-fos mRNA was not detected in either cell line, the role of AP-1 (c-fos/c-jun) heterodimers in

Table 1. — *Expression of progesterone receptor mRNA, detected by RT-PCR using FAM labeled primer, and progesterone protein, detected using PR monoclonal antibodies and the Ventana automatic staining system in various human cells. NT = not tested. ND = not detected.*

Cells	Phenotype	PR mRNA	Multiple transcripts	PR protein
B Cells				
R-CLL	B-CLL	NT	NT	ND
RPMI-8226	Myeloma	+	+	+
U226	myeloma	+	+	+
HS-1	HTLV-1+	NT	NT	ND
729PH6neo	HTLV-2+	NT	NT	ND
SRIH-BATL	HTLV-1+	NT	NT	ND
T Cells				
MOT	HTLV-2+CD4+	+	+	+
HUT-102	HTLV-1+CD4+	ND	ND	NT
SRIC-ATL	HTLV-1+CD4+	NT	NT	+
FC36.22	HTLV-1+CD8+	NT	NT	+
JURKAT	CD4+	ND	ND	ND
PHA treated normal CD4+ and CD8+ T	Mitogen activated T cells	ND	ND	NT
Myeloid Cells				
U937	Monoblast	+	+	+
THP-1	Monoblast	+	+	NT
KG-1	Myeloblast	NT	NT	+
K562	CML-blast	+	+	+
HL-60	Myeloblast	ND	ND	NT
Other				
PMNC	Normal lymphocytes and monocytes	ND	ND	NT
MCF-7		+	+	+

cytokine modulation was not investigated further. Focus was directed to NFκB and GR.

Modulation of NFκB and GR activity by progesterone: Results of transient transfections with reporter plasmid for NFκB in MOT and U937 cells, expressed in relative light units, are given in Figures 3a and 3b, respectively. Treatment with 5-50 μM progesterone significantly inhibited the activity of this transcription factor, with effects as early as four to six hours, and persisting to 24-48 hours, the endpoint of the study. The monoblast cell line U937, which transfects with greater efficiency than MOT T cells, showed very high constitutive NFκB activity. This was nearly abolished by treatment with 50 μM P for 18-48 hours. Concentration dependence was also shown with 5 μM P. These results strongly support that progesterone down-regulates the immune response, including cytokine production, by decreasing the transcription activity of NFκB in human immune cells. Results of transient transfections with the reporter plasmid for GR are given in Figure 4a for MOT and Figure 4b for U937. As expected, GR activity was greatly increased by 5 μg/ml dexamethasone in MOT cells by 24 hours. Treatment of cells with 50 μM P, however, significantly inhibited GR activity, and when combined with dexamethasone, prevented GR activation by dexamethasone. Concentration dependence was again observed, but 5 μM P also attenuated GR activation with dexamethasone. In U937 cells, P at 50 μM significantly inhibited GR. These results support that progesterone, via one or more of the PR isoforms can modulate the activity of the key transcription factors – GR and NFκB – that control the immune response, and profoundly alter immune cell responses.

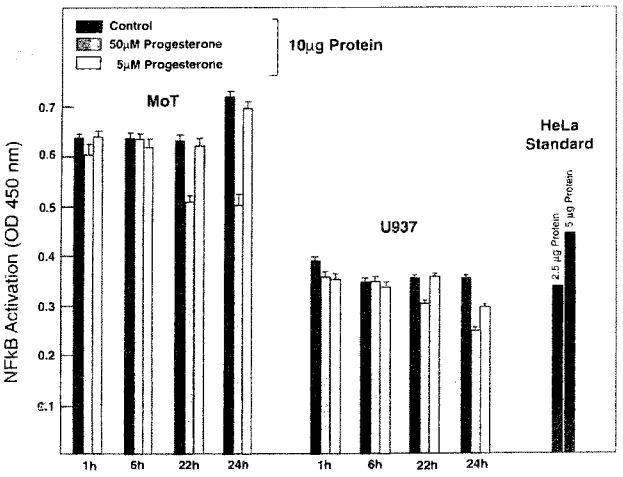

Figure 5. — Modulation by P of NF-κB p65 activation in MOT and U937 cells as measured by the ELISA-based kit from Active Motif. Whole extracts containing 10 μg protein from MOT and U937 cells, and 2.5 and 5 μg protein from activated HeLa cells (standards) were used.

The effect of P on the activated form of NF-κB p65 in MOT and U937 cells (Figure 5) as determined by the ELISA-based kit from Active Motif demonstrates the reproducibility of this procedure and confirms the above results showing significant inhibition of NF-κB p65 activation by 50 μM P at about a 22-24 hour incubation with the drug.

Discussion

Using cell lines, MOT and U937, which down-regulate their cytokine production in the presence of physiological P, we have been able to demonstrate the presence of progesterone receptor protein and mRNAs, and determine that immunosuppressive effects of P may be due in part to NFκB inhibition.

To address the controversial issue of progesterone receptor presence we utilized the classic techniques of immunohistochemistry, standard RT-PCR, and also Gene Scan. The immunohistochemistry clearly demonstrated PR protein expression in the nucleus of most but not all MOT and U937 cells, and strongly stained PR-positive control progesterone sensitive breast cancer tissue and MCF-7 breast carcinoma cell lines. Individual cell differences in PR expression, even within one cell line, could allow the overall sensitivity to progesterone to depend on the number of positive cells and the number of functional PR receptors on the cells. This may vary from culture to culture, and be affected by hormonal milieu, as estrogen treatment appeared to up-regulate expression.

The standard RT-PCR involving ethidium bromide staining of products failed to demonstrate mRNA for the progesterone receptor in either MOT or U937, but was positive for breast and vaginal epithelial lines, validating the primers used. The results of Gene Scan, a significantly more sensitive technique, with the same primer sequence used for RT-PCR, but FAM labeled, clearly demonstrate the presence of PR mRNA in MOT, U937, other B, T and myeloid lines, with massive amounts in MCF-7 (Table 1).

The Gene Scan also revealed for the first time the presence of multiple transcripts, in various amounts, suggesting the existence of possible alternative splicing or PR initiation sites in human leukocytes, as has been reported for other progesterone sensitive cells [34, 40, 41]. Thus, possible agonist/antagonist effects with progesterone treatment, depending on the relative amounts of each receptor isoforms transcribed and expressed, may occur. The primers used in these experiments detect both PR-α and PR-β, which could be two of the isoform species detected. These results clearly demonstrate an enormous complexity to progesterone-immune cell interactions, and help to explain the great diversity of progesterone effects, as well as possible sources of prior confusion or conflicting results regarding PR expression, and support the need for highly sensitive assay for PR detection.

The increase in PR mRNA with estradiol treatment in MOT and U937 further supports the modulation by other hormones in the milieu. Thus, culture conditions or systemic or local alterations in the concentration of other sex hormones could profoundly affect progesterone action. This may be an important mechanism of the systemic immunosuppressant effect during pregnancy, as elevated serum estrogens could increase leukocyte sensitivity to progesterone via up-regulation of PR receptors. At the fetomaternal interface, with its extremely high local concentration of progesterone and estrogens, it may be important to fetal tolerance. There is evidence that the P receptor on gamma/delta T cells is a relatively insensitive P receptor that may require high local concentrations to allow progesterone-induced blocking factor (PIBF) expression. This in turn could suppress NK cell activity only at the maternofetal interface where such high concentrations of P are reached. Thus, only local immune tolerance is induced.

We used standard RT-PCR and characterized the relevant transcription factor profiles of MOT and U937, and determined the effect of progesterone on the steady state RNA levels for these factors. The critical component of AP-1, c-fos, was not expressed, supporting the exclusion of this factor in cytokine modulation. Also, as progesterone did not detectably alter the mRNA levels for components of NFκB (NFκB p65, p52, p50) and IκB-α, we concluded the mechanism of immunomodulation was unlikely to be at the transcription level for this protein.

The molecular techniques of transient transfection with SEAP reporter plasmid for NFκB enabled us to quantitatively determine binding activity and transcription activation by NFκB. These studies showed for the first time that NFκB activity was profoundly suppressed by progesterone in U937 and MOT cells, and is likely a critical mechanism of immunomodulation in these cell types. Quantitation of activated NF-κB p65 in whole cell extracts using ELISA-based Trans AM p65 kit also showed its inhibition by P in MOT and U937 cells. Suppression of cytokine production by NFκB inhibition would help to explain the ability of P to influence the course of inflammatory/autoimmune and infectious diseases, as is observed in some patients.

U937 and MOT cells transiently transfected with SEAP reporter plasmid for GR, upon treatment with dexamethasone, showed, as expected, an increase in GR transcription activity. However, these cells, when treated with progesterone, showed inhibition of GR transcription activity. This could have resulted from binding of PR to an antagonist binding site on GR reportedly located topographically close to the glucocorticoid-binding site. However, the results may also suggest that complex interactions between PR and other regulators of transcription are occurring, with mixed agonist-antagonist effects possibly due to the presence of different PR isoforms. Understanding these effects will require further studies at the molecular level.

Conclusions

In conclusion, physiologic concentrations of progesterone are immunosuppressive and inhibit chemokine and cytokine production by human T, myeloid, and B cells, but there are significant differences in sensitivity between different lines, even of the same major lineage. Some, but not all, human T, B, and myeloid cells express mRNAs of variable fragment sizes and amounts for progesterone receptors. These, if transcribed and the proteins expressed, may influence the overall effect of progesterone on immune cell behavior. Some human T, B, and myeloid cells clearly express progesterone receptor proteins that may bind ligand, but individual cells, even of the same line in the same culture, may have different levels of expression. Culture conditions, including concentration of other sex hormones such as estradiol, may alter progesterone receptor expression, and the effects of progesterone exposure. The molecular mechanism of cytokine suppression likely involves NFκB, as its activity is nearly abolished by treatment with progesterone. However, the inhibition of GR activity suggests that very complex interactions between PR and other regulators of transcription are occurring, with agonist/antagonist effects possibly due to PR isoforms. These experiments reveal potential sources of prior conflict in the literature, but establish that PR mRNA and protein can be detected in activated T, B, and myeloid cells using highly sensitive procedures. The results further highlight the enormous diversity and complexity of interaction between sex hormones, transcription factors, and cells of the human immune system, influencing reproduction, infection, autoimmunity, and neoplasia.

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